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Predominant localization of phospho*enol*pyruvate carboxykinase mRNA in the periportal zone of rat liver parenchyma demonstrated by in situ hybridization

Heidrun Bartels, Helga Linnemann and Kurt Jungermann

Institut für Biochemie, Georg-August-Universität, Humboldtallee 23, D-3400 Göttingen, FRG

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In rat liver parenchyma, expression of the phospho*enol*pyruvate carboxykinase (PEPCK) gene was studied by Northern blot analysis with a biotinylated cRNA probe and the zonal localization of PEPCK mRNA was demonstrated by in situ hybridization with a radiolabelled cRNA probe. During the feeding period at night, overall PEPCK mRNA levels were low and PEPCK mRNA was detected only in small areas of the periportal zone. At the beginning of the light period (7 am) the overall PEPCK mRNA level began increasing and the periportal areas containing PEPCK mRNA broadened. The maximum of the total abundance and of the area with high levels of PEPCK mRNA was reached at noon. Fasting for 24–72 h did not cause further significant alterations in the level or localization of PEPCK mRNA. The present data are in line with previous findings of the predominant localization of PEPCK activity and enzyme protein in periportal hepatocytes. They suggest that the heterogeneous expression of the PEPCK gene in rat liver is regulated at the pretranslational level.

Phospho*enol*pyruvate carboxykinase; Hybridization; mRNA; Metabolic zonation; (Periportal zone, perivenous zone, Rat liver)

1. INTRODUCTION

Periportal hepatocytes contain higher activities and immunoreactive quantities of the gluconeogenic and perivenous cells of the key glycolytic enzymes [1–7]. On the basis of enzyme distribution, the model of 'metabolic zonation' proposes that glucose release via glycogenolysis and gluconeogenesis and the consequent formation of glycogen via the indirect gluconeogenic route are preferentially catalyzed by periportal hepatocytes, whereas glucose uptake for glycogen synthesis via the direct route and glycolysis are mainly mediated by perivenous cells [1]. Using perfused rat liver, direct evidence in support of the model has recently been obtained [8]. The activities of the key gluconeogenic enzymes phospho*enol*pyruvate carboxykinase (PEPCK) and fructose-1,6-bisphosphatase as demonstrated by microdissection techniques [2,6] and the enzyme proteins as shown immunohistochemically [3,7] are located predominantly in the periportal zone. Conversely, the activities [2,4,6] and enzyme proteins [5,7] of the key glycolytic enzymes pyruvate kinase type L (PK_L) and glucokinase are situated in the perivenous zone. Little information is available on the zonal distribution of mRNA of key enzymes of carbohydrate metabolism. Only one study indicated that the PK_L mRNA was distributed homogeneously in the parenchyma [9] in contrast to the perivenous maximum of enzyme activity and protein [2,4,5]. It was therefore the goal of the present study to elucidate by in situ hybridization the zonal distribution of PEPCK mRNA and to determine whether the heterogeneous expression of the PEPCK gene was caused primarily at the pretranslational, translational or posttranslational

Correspondence address: H. Bartels, Institut für Biochemie, Georg-August-Universität, Humboldtallee 23, D-3400 Göttingen, FRG

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level. A preliminary report of some findings has appeared [10].

2. MATERIALS AND METHODS

2.1. *Materials*

All chemicals were of the highest purity available and were obtained from commercial sources. The plasmid pSP-PCK was a gift from Drs G. Schütz and W. Schmid (Heidelberg) and was constructed using a 1.2 kb cDNA fragment of PEPCK provided by Dr Granner (Nashville).

2.2. *Animals and preparation of liver samples*

Male Wistar rats (Winkelmann, Borchen) were used throughout. Animals were kept on a 12 h day–12 h night rhythm and were allowed water ad libitum, but access to food (standard diet 1320, Altromin, Lage) was limited only during the night (7 pm–7 am). Rats were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg body wt). Blood was removed from livers by brief perfusion with 0.9% NaCl. Liver samples (~500 mg) used to prepare sections for in situ hybridization were frozen in isopentane at −30°C and stored at −70°C until use. Samples for total RNA preparation were frozen in liquid N_2, crushed and extracted immediately.

2.3. *In situ hybridization*

The procedures used were based mostly on the protocol of Holland [11].

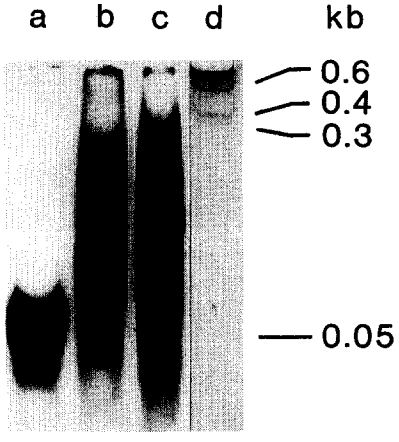

Fig.1. Size analysis of cRNA probes for in situ hybridization of PEPCK mRNA. Radiolabelled RNA probes of length 1.2 kb complementary (antisense) (b) and non-complementary (sense) (c) to PEPCK mRNA were subjected to alkaline hydrolysis and then analyzed by electrophoresis in an 8% polyacrylamide gel under denaturing conditions with 7 M urea. Standards were a 0.05 kb ^{35}S-labelled RNA (a), which was generated by in vitro transcription with T_7 polymerase from a *Hin*dIII-linearized pBluescript-PCK, and an RNA ladder (d) with fragments of 0.3, 0.4, 0.6, 1.0 and 1.6 kb in length (purchased from Boehringer, Mannheim). The RNA ladder was stained with methylene blue. Autoradiography was performed for 36 h at −70°C. The RNA ladder was stained with methylene blue.

2.3.1. Preparation of hybridization probes

The *Pst*I cDNA fragment of phospho*enol*pyruvate carboxykinase of plasmid pSP-PCK was subcloned into the *Pst*I restriction site of a Bluescript vector (pBS). The polylinker of this plasmid is flanked by promotors for T_7 and t_3 RNA polymerase. The complementary antisense RNA probe was generated from a *Hin*dIII linearized pBS-PCK with T_3 polymerase, the non-complementary sense RNA probe from a *Sac*I-linearized pBS-PCK with T_7 polymerase. The sense transcript was used to determine unspecific hybridization. Transcripts were labelled with uridine 5′-[α-^{35}S]thiotriphosphate (Amersham Buchler, Braunschweig). The original transcription reaction described by Holland [11] was modified according to the Gibco/BRL manual to prepare high specific activity cRNA probes using T_7 and T_3 polymerase. Each transcription reaction at 37°C yielded 0.1–0.15 μg RNA with a specific activity of 5–10 × 10^8 cpm/μg. Lower temperatures reduced the yield significantly. Transcripts were subjected to alkaline hydrolysis with Na_2CO_3 buffer (pH 10.2) for 80 min at 60°C to reduce the original length of 1.2 kb to about 0.1–0.2 kb. Transcripts were precipitated with 96% ethanol, dried under vacuum and dissolved in 50% deionized formamide/10 mM dithioerythritol (DTE) and stored at −20°C.

2.3.2. Section pretreatment and hybridization procedure

Cryostat sections (8 μm) were mounted on polylysine-coated slides, air-dried and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 10 min. After fixation, sections were washed in PBS, immersed in 0.25% (v/v) acetic anhydride in 0.1 M triethanolamine buffer (pH 8.0) for 10 min, dehydrated in graded ethanol and used for hybridization. The hybridization mixture contained in 1 × Denhardt's solution (0.02% each of bovine serum albumin, polyvinylpyrrolidone and Ficoll) and 1 × 'salts' (0.3 M NaCl, 0.01 M Tris-HCl, 0.01 M sodium phosphate; pH 6.8), 50% (v/v) deionized formamide, 10% dextran sulfate, 10 mM DTE and 0.7 μg/μl freshly denatured salmon sperm DNA. Complementary and

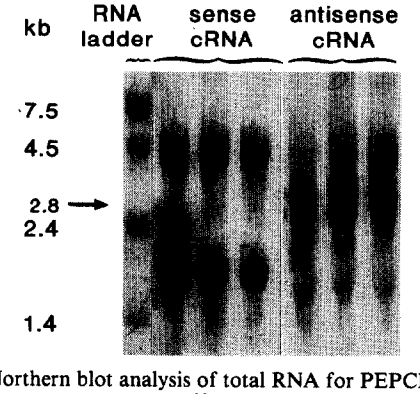

Fig.2. Northern blot analysis of total RNA for PEPCK mRNA using the alkali-shortened ^{35}S-labelled sense and antisense cRNA probes. Total RNA was prepared and 10 μg of each were separated by electrophoresis in a 1.5% agarose gel under denaturing conditions, and subsequently blotted and hybridized to the radiolabelled probes, the concentration of which was 2.5 ng/ml hybridization mixture. Autoradiography was performed for 3 days at room temperature. The RNA ladder was stained with methylene blue.

non-complementary transcripts were diluted in hybridization solution to about 5×10^4 cpm/μl, corresponding to 0.05–0.1 ng radiolabelled RNA/μl. Each section was covered with 40 μl hybridization mixture. Hybridization was performed overnight at 55°C in a chamber humidified with 50% deionized formamide/1 × salts. All post-hybridization procedures were carried out without formamide. Sections were washed in 2 × NaCl/citrate (0.3 M NaCl, 0.03 M sodium citrate; pH 7.4) for 1 h at 50°C, then immersed in 0.5 M NaCl in TE buffer (0.01 M Tris, 1 mM EDTA; pH 7.6) for 15 min at 37°C, and treated with RNase (20 μg/ml, 0.5 M NaCl in TE buffer) for 30 min at 37°C. RNase was removed by immersion of sections in 0.5 M NaCl in TE buffer for 30 min at 37°C. Finally, sections were washed four times (30 min each) in 2 × NaCl/citrate at room temperature. Sections were dried in graded ethanol and dipped in Kodak NTB 2 emulsion diluted 1:2 with 2% (v/v) glycerol. Autoradiography was performed at 4°C for 7 days.

2.4. *Histochemistry*

Succinate dehydrogenase (SDH) was visualized histochemically as indicated by Lojda et al. [12].

2.5. *Preparation and Northern blot analysis of total RNA*

Total RNA was prepared, separated by electrophoresis and blotted onto nitrocellulose; PEPCK mRNA was hybridized as indicated either to ^{35}S-labelled antisense or sense cRNA or to a biotinylated antisense cRNA probe, visualized and finally quantified as described by Christ et al. [13].

2.6. *Determination of PEPCK activity*

Enzyme activity was measured in the 100000 × g supernatant of a 1:10 liver homogenate according to Seubert and Huth [14].

3. RESULTS

3.1. *Characterization of hybridization probes*

A length of 0.1–0.2 kb is recommended for most in situ hybridization procedures to achieve efficient hybridization [11,15,16]. Therefore, the 1.2 kb single-stranded ^{35}S-labelled antisense and sense RNA probes generated for in situ hybridiza-

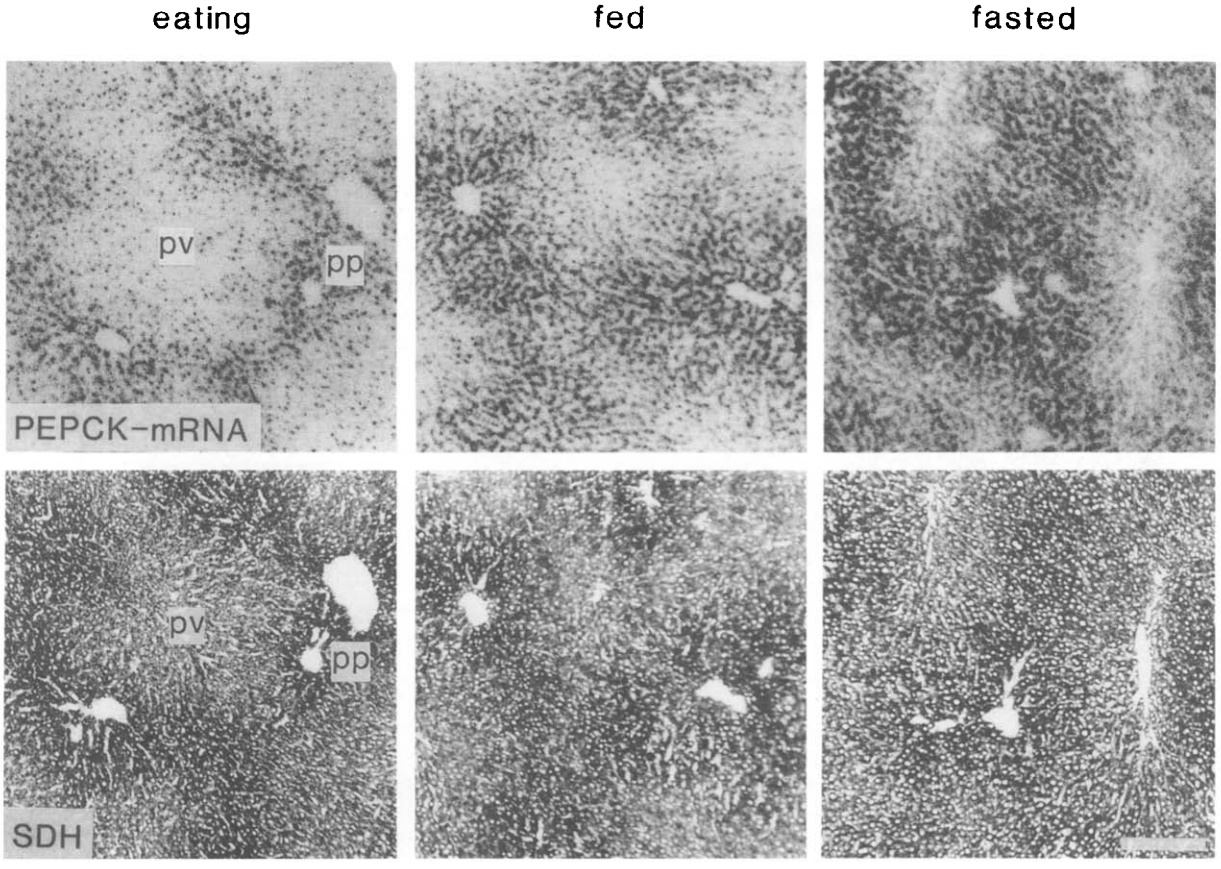

Fig.3. Distribution of PEPCK mRNA in liver parenchyma of feeding, fed and 24 h fasted rats. In situ hybridization of PEPCK mRNA was performed with alkali-shortened ^{35}S-labelled antisense cRNA probes. For comparison the periportal marker enzyme succinate dehydrogenase (SDH) was demonstrated histochemically in parallel sections. PEPCK mRNA (high grain density) and SDH (dark precipitates) show higher levels in the periportal (pp) vs perivenous (pv) zone. Feeding, 3 am; fed, noon; 24 h fasted, no food from 7 am to 7 am. Bar, 200 μm.

tion of PEPCK mRNA were shortened by alkaline hydrolysis to a final length of 0.05–0.3 kb (fig.1).

Northern blot analysis (fig.2) showed that the shortened antisense transcripts hybridized specifically to the 2.8 kb PEPCK mRNA. The shortened sense transcripts did not hybridize specifically to any mRNA. This result confirmed that the antisense and sense transcripts were reliable hybridization probes. The density of the hybridization signal obtained with the shortened radiolabelled antisense transcripts (fig.2) was measured by video-densitometry and compared to the signal obtained with the 1.2 kb biotinylated antisense cRNA probe. The results were identical (not shown) and corroborated the reliability of the ^{35}S-labelled shortened transcripts as hybridization probes.

3.2. *Zonal distribution of PEPCK mRNA*

The distribution of PEPCK mRNA in rat liver parenchyma was investigated under different feeding conditions: during the normal day-night rhythm and after 24–72 h starvation. SDH served as marker enzyme for the periportal zone [1]. PEPCK mRNA was distributed heterogeneously within rat liver parenchyma under all conditions: periportal hepatocytes contained greater amounts of PEPCK mRNA than perivenous cells (figs 3,4). The pattern of PEPCK mRNA distribution within the periportal zone varied according to the feeding state of the rats: During the night, when rats were feeding, PEPCK mRNA was restricted to fewer cells within the periportal zone than at noon, when the rats were in the fed state. At this stage of the feeding rhythm PEPCK mRNA was distributed across the periportal and intermediate zones (fig.3). Apparently, the PEPCK mRNA distribution changed dynamically between 3 am and noon; yet, upon transition to prolonged starvation for 24 h (fig.3) and 72 h (not shown) the pattern of PEPCK mRNA distribution at noon was not significantly altered further.

The abundance of PEPCK mRNA in perivenous hepatocytes was low, specially in those cells surrounding the terminal venule (fig.3), but not zero as demonstrated by analyzing sections at higher magnification (fig.4). The hybridization density with antisense probes in perivenous cells (fig.4B) was clearly less than in periportal cells (fig.4A), but still significantly higher than the unspecific hybridization density with sense probes (fig.4C).

3.3. *PEPCK mRNA abundance and PEPCK activity*

During the feeding period in the dark, the total amount of PEPCK mRNA in liver was low; it began to increase with the beginning of the fasting period in the light, reaching a maximum at noon.

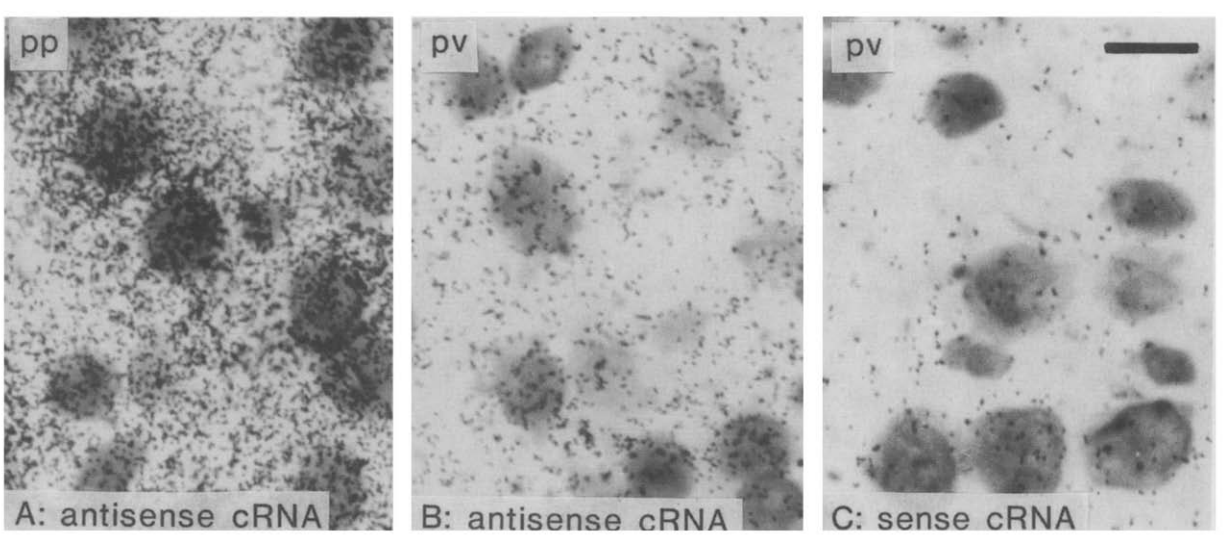

Fig.4. Level of PEPCK mRNA in periportal and perivenous areas. Liver sections were hybridized with alkali-shortened ^{35}S-labelled antisense or sense cRNA probes for PEPCK. The density of silver grains using the antisense probe was clearly higher in the periportal (pp) than in the perivenous (pv) zone. The density of grains found in perivenous hepatocytes with antisense cRNA was clearly reduced further when unspecific binding was determined with sense cRNA. Nuclei were stained with hematoxylin. Bar, 10 μm.

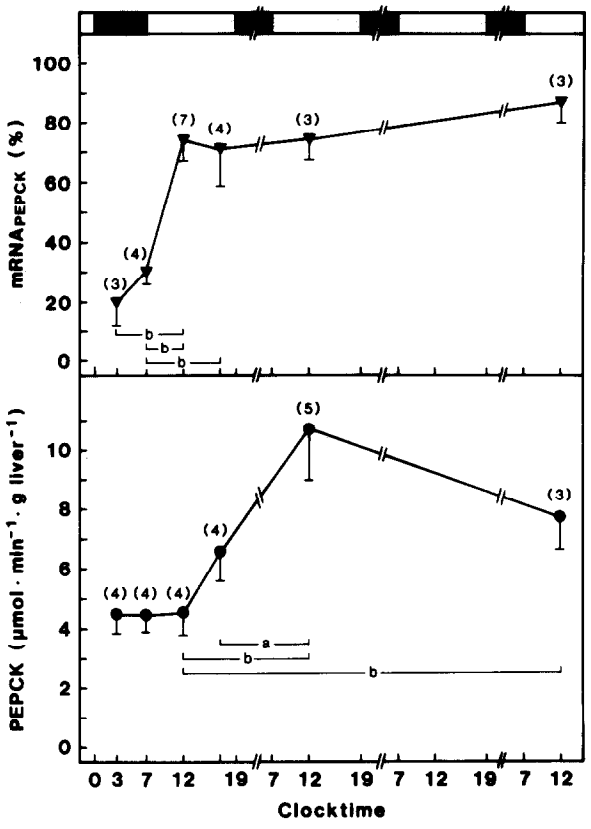

Fig.5. Time course of total mRNA level and activity of PEPCK during the transition from a normal feeding cycle to starvation. Animals had access to food up to 7 am during the first night (dark period) only. PEPCK mRNA abundance was determined with the biotinylated antisense cRNA probe and PEPCK activity via a standard test. Values are means ± SE for the number of animals given in parentheses. Statistical analysis was performed by Student's *t*-test: [a] $p < 0.05$, [b] $p < 0.01$.

This level remained unaltered within the subsequent hours and did not change significantly during prolonged starvation up to 72 h (fig.5). In comparison to PEPCK mRNA, PEPCK activity was also low during the dark period, however, it began increasing with a lag reaching a maximum after 24 h starvation (fig.5).

4. DISCUSSION

4.1. *Transcriptional or translational regulation of zonal gene expression*

Periportal hepatocytes contained greater amounts of PEPCK mRNA than perivenous cells (figs 3,4). The predominant periportal localization was maintained during not only the normal feeding rhythm but also fasting and starvation. This result is in accord with the higher activity of PEPCK and greater amount of enzyme protein in the periportal zone as demonstrated in [2,3]. The present findings therefore suggest that the zonal heterogeneity of gluconeogenic PEPCK is controlled primarily at the pretranslational level, i.e. either the rate of transcription of the PEPCK gene is higher and/or the degradation of PEPCK mRNA is lower in the periportal vs perivenous zone.

Recently, it was reported that mRNA of the key glycolytic enzyme PK_L was distributed homogeneously within rat liver parenchyma [9], although the enzyme activity and protein were shown to be preferentially localized in perivenous hepatocytes [2,4,5]. These results suggest that the zonal heterogeneity of PK_L, in contrast to that of PEPCK, is due to regulation at the translational or post-translational level, i.e. the rate of synthesis of PK_L using the same amount of message is higher and/or the degradation of PK_L-protein is lower in the perivenous than in the periportal zone. Perhaps the site of regulation for heterogenous zonal expression of gluconeogenic and glycolytic enzymes is generally different; however, a conclusion is not possible at present, since no further studies on the zonal localization of mRNA of other zonated key enzymes of gluconeogenesis and glycolysis such as fructose-1,6-bisphosphatase [6,7], glucose-6-phosphatase [17] or glucokinase [6,7] have been reported.

The mRNAs of carbamoyl-phosphate synthetase (CAPS) and glutamine synthetase (GS), key enzymes of ammonia detoxification via urea and glutamine formation, were shown to be distributed reciprocally within the parenchyma as were the enzyme proteins [18–20]: GS mRNA was restricted to only a few distal hepatocytes within the perivenous zone around the terminal venules whereas CAPS mRNA was located in all hepatocytes of the periportal zone and in the proximal cells of the perivenous zone, i.e. in cells not containing GS mRNA. The mRNA of phenobarbital-inducible cytochrome P450b and P450e showed higher levels in the perivenous area in correlation with the greater level of enzyme protein in this zone [21,22]. These studies on the distribution of CAPS, GS and cytochrome P450 mRNAs suggest that pretranslational regulation

may be the most widespread mechanism for the zonal heterogeneity of gene expression.

The zonal localization of mRNA of serum proteins synthesized in the liver such as albumin [23–25] and α-fetoprotein [26] is unclear; for albumin mRNA, homogeneous [23,26] as well as heterogeneous [24,25] distribution with a periportal maximum has been reported, while for α-fetoprotein mRNA [26] uniform distribution has been observed.

4.2. *Factors involved in the regulation of zonal gene expression*

The key enzymes of carbohydrate metabolism such as PEPCK and PK_L and those of xenobiotic metabolism, e.g. cytochrome P450, show 'dynamic' zonation, i.e. their zonal activities and quantities change according to the metabolic state. Differential activation of their genes appears to be controlled primarily by the hormonal and probably nervous system and by substrate supply [1]: induction of PEPCK by glucagon via cAMP [27–29] under the permissive action of glucocorticoids [29] is antagonized by insulin [13,27,29] and extracellular adenosine [30] and modulated by physiological oxygen tensions [31]. The increase in glucagon/insulin [13] and the glucagon/adenosine [29] ratios as well as the decrease in oxygen tension [30] during the passage of blood through the liver could therefore play an important role in the predominant expression of the PEPCK gene in the periportal zone [1]. Since a significant periportal to perivenous gradient of the sinusoidal phenobarbital concentration was not observed, a greater degree of trapping of phenobarbital by perivenous cells could contribute to the preferential induction of cytochrome P450 by phenobarbital in the perivenous zone [22].

In contrast, the key enzymes of ammonia detoxification such as CAPS and GS show a 'stable' zonation, i.e. their distributions remain unaltered, irrespective of the metabolic state. Therefore, the activation of their genes does not appear to be regulated by the hormonal or nervous system or substrate supply; it may be controlled by the acinar architecture [18], or cell-cell and cell-matrix interactions [19].

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REFERENCES

[1] Jungermann, K. (1988) Semin. Liver Dis. 8, 329–343.
[2] Guder, W.G., Schmidt, U., Funk, B., Weiss, S. and Pürschel, S. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1793–1800.
[3] Andersen, B., Nath, A. and Jungermann, K. (1982) Eur. J. Cell Biol. 28, 47–53.
[4] Zierz, S., Katz, N. and Jungermann, K. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 1447–1453.
[5] Chatzipanagiotou, S., Nath, A., Vogt, B. and Jungermann, K. (1985) Biol. Chem. Hoppe-Seyler 366, 271–280.
[6] Katz, N., Teutsch, H.F., Jungermann, K. and Sasse, D. (1977) FEBS Lett. 83, 272–276.
[7] Lawrence, G.M., Jepson, M.A., Trayer, I.P. and Walker, D.G. (1986) Histochem. J. 18, 45–53.
[8] Bartels, H., Vogt, B. and Jungermann, K. (1988) Histochemistry 29, 253–260.
[9] Lamas, E., Kahn, A. and Guillouzo, A. (1987) J. Histochem. Cytochem. 35, 559–563.
[10] Bartels, H., Linneman, H. and Jungermann, K. (1989) Z. Gastroenterol. 27, 17.
[11] Holland, P. (1986) in: Manipulating the Mouse Embryo (Hogan, B. et al. eds) pp.228–242, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[12] Lojda, Z., Gossrau, R. and Schiebler, T.H. (1976) Enzymhistochemische Methoden, Springer, Berlin.
[13] Christ, B., Nath, A., Bastian, H. and Jungermann, K. (1988) Eur. J. Biochem. 178, 373–379.
[14] Seubert, W. and Huth, W. (1965) Biochem. Z. 343, 167–191.
[15] Lawrence, J.B. and Singer, R.H. (1985) Nucleic Acids Res. 13, 1777–1799.
[16] Singer, R.H., Lawrence, J.B. and Villnave, C. (1986) BioTechniques 4, 230–250.
[17] Katz, N., Teutsch, H., Sasse, D. and Jungermann, K. (1977) FEBS Lett. 76, 226–230.
[18] Moorman, A.F.M., De Boer, P.A.J., Geerts, W.J.C., Van de Zande, L., Lamers, W.H. and Charles, R. (1988) J. Histochem. Cytochem. 36, 751–755.
[19] Gebhardt, R., Ebert, A. and Bauer, G. (1988) FEBS Lett. 241, 89–93.
[20] Smith, D.D. and Campbell, J.W. (1988) Proc. Natl. Acad. Sci. USA 85, 160–164.
[21] Schwarz, M., Peres, G., Buchmann, A., Friedberg, T., Waxman, D.J. and Kunz, W. (1987) Carcinogenesis 8, 1355–1357.
[22] Wojcik, E., Dvorak, C., Chianale, J., Traber, P.G., Keren, D. and Gumucio, J.J. (1988) J. Clin. Invest. 82, 658–666.
[23] Bernuau, D., Poliard, A., Tournier, I., Sala-Trepat, J. and Feldmann, G. (1985) Cell Biol. Int. Rep. 9, 31.
[24] Schwarz, M., Peres, G., Beer, D.G., Maor, M., Buchmann, A., Kunz, W. and Pitot, C.H. (1986) Cancer Res. 46, 5903–5912.

[25] Evarts, R.P., Nagy, P., Marsden, E. and Thorgeirsson, S.S. (1987) Cancer Res. 47, 5469–5475.
[26] Bernuau, D., Poliard, A. and Feldmann, G. (1988) Hepatology 8, 997–1005.
[27] Sasaki, K., Cripe, T.P., Koch, S.R., Andreone, T.L., Petersen, D.D., Beale, E.G. and Granner, D.K. (1984) J. Biol. Chem. 259, 15242–15251.
[28] Buchler, W., Walter, U., Jastorff, B. and Lohmann, S.M. (1988) FEBS Lett. 228, 27–32.
[29] Probst, I. and Jungermann, K. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 1447–1453.
[30] Christ, B., Löhne, R., Schmidt, H. and Jungermann, K. (1987) Biol. Chem. Hoppe-Seyler 368, 1579–1587.
[31] Nauck, M., Wölfle, D., Katz, N. and Jungermann, K. (1981) Eur. J. Biochem. 119, 657–661.